



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/62, C07K 14/035, 14/47, C12N 15/79		A1	(11) International Publication Number: WO 98/32866
			(43) International Publication Date: 30 July 1998 (30.07.98)
(21) International Application Number: PCT/GB98/00207 (22) International Filing Date: 23 January 1998 (23.01.98) (30) Priority Data: 9701363.5 23 January 1997 (23.01.97) GB 9716398.4 1 August 1997 (01.08.97) GB (71) Applicant (for all designated States except US): MARIE CURIE CANCER CARE [GB/GB]; 28 Belgrave Square, London SW1X 8QC (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): O'HARE, Peter, Francis, Joseph [GB/GB]; 15 Beatrice Road, Oxted, Surrey RH8 0TL (GB). ELLIOTT, Gillian, Daphne [GB/GB]; Flat 4, 123 Station Road East, Oxted, Surrey RH8 0QT (GB). (74) Agents: KREMER, Simon, M. et al.; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.	
(54) Title: FUSION PROTEINS FOR INTRACELLULAR AND INTERCELLULAR TRANSPORT AND THEIR USES			
(57) Abstract			
Coupled polypeptides and fusion polypeptides for intracellular transport, and their preparation and use, include (i) an amino acid sequence with the transport function of herpesviral VP22 protein (or homologue, e.g. from VZV, BHV or MDV) and (ii) another protein sequence selected from (a) proteins for cell cycle control; (b) suicide proteins; (c) antigenic sequences or antigenic proteins from microbial and viral antigens and tumour antigens; (d) immunomodulating proteins; and (e) therapeutic proteins. The coupled proteins can be used for intracellular delivery of protein sequences (ii), to exert the corresponding effector function in the target cell, and the fusion polypeptides can be expressed from corresponding polynucleotides, vectors and host cells.		<p>The diagram shows a circular plasmid vector labeled p4953ep+10 with a size of 6.5kb. Starting from the top and moving clockwise, the features are: a Bam HI restriction site, the p53 gene, another Bam HI site, an epitope, a poly(A) site, a CMV promoter, a Bgl II site, and the VP22 gene. The VP22 gene is represented by a hatched segment.</p>	

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakistan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

FUSION PROTEINS FOR INTRACELLULAR AND INTERCELLULAR TRANSPORT AND THEIR USES

Field of the Invention:

5 The present invention concerns improvements, modifications and developments in relation to transport proteins, intracellular transport and their applications. In particular embodiments, the invention relates to fusion proteins comprising transport proteins comprising sequences from herpesviral VP22 or from homologues or fragments thereof together with sequences from other proteins; and to methods for their preparation and use. In particular embodiments, the invention
10 relates to fusion proteins for cell cycle control, and to materials and methods for their preparation and their use. In particular examples the invention relates to fusion proteins having both mammalian p53 functionality and herpesviral VP22 functionality. Other aspects of the invention will be apparent from the description and claims.

15 Background of the invention, and prior art:

Relevant to the present application is the inventors' own earlier international patent application WO 97/05265 (O'Hare and Elliott) (published after the priority date claimed for this application), which relates to VP22 protein and its properties and
20 uses. Similarly the inventors' paper (Elliott and O'Hare (1997), in Cell, vol 88 pp 223-233 (1997), relates to intercellular trafficking and protein delivery by a herpesvirus structural protein. Both these documents are hereby incorporated in their entirety by reference and made an integral part of this disclosure.

25 The inventors have shown that the HSV-1 virion protein VP22 possesses an unusual intercellular trafficking mechanism, an effect particularly described in specification WO 97/05265. VP22 is a 38kDa protein which in primary-expressing transfected mammalian cells is located predominantly in the cytoplasm where it associates with cellular microtubules (see accompanying drawing, Fig 1b). However
30 a remarkable property of VP22 is its ability to spread throughout a monolayer of non-expressing cells. VP22 is transported from the cytoplasm of an expressing cell into neighbouring cells where it accumulates in the nucleus (Fig 1b). The mechanism of this transport is still incompletely understood, but has been shown to be via a golgi-independent pathway and may utilise the actin cytoskeleton. HIV-1 Tat (Ensoli et al.,
35 1993, Fawell et. al., 1994) and a small number of other non-viral proteins (Jackson

et al., 1992) have been attributed with intercellular trafficking properties, but none appears to demonstrate this phenomenon as strikingly as VP22. A further important property of VP22 is that when applied exogenously to the medium of an untransfected cell monolayer, it can be taken up by those untransfected cells where it accumulates in the cell nucleus.

The prior art generally includes a variety of antigens, immunomodulating proteins, proteins that are conditionally cytotoxic or lethal upon administration (to a cell containing them) of a corresponding drug or activator compound, proteins for cell cycle control, and other therapeutic and diagnostic proteins, especially in the forms of protein and polynucleotide sequences enabling genetic manipulation by standard techniques. References to some examples of these materials are given below.

For example, among cell cycle control proteins, protein p53 is known as a tumour suppressor. p53 is a 53kDa nuclear phosphoprotein (Fig 1c). Wild-type and mutant p53 proteins have been expressed by means of recombinant vaccinia viruses, (Ronen et al., Nucleic Acids Research, 20:3435-3441, 1992). p53 functions to regulate cell cycle progression and under conditions of DNA damage through a complex signal transduction mechanism can induce cell cycle arrest or apoptosis (Levine 1997). Failure to synthesize p53, or more commonly synthesis of a mutated form of the protein can result in uncontrolled cell proliferation and tumour formation. It has been shown by several groups that exogenous addition of functional wild type p53 can promote cell cycle arrest and/or apoptosis resulting in tumour regression with examples including cervical carcinomas (Hamada et al., 1996) and breast cancer xenografts (Nielsen et al., 1997). A number of p53 delivery systems have been utilised in vivo and in vitro such as intravenous injection of a p53:liposome complex (Kumar et al., 1997), direct transfection (Zheng et al., 1996) and adenoviral mediated transfer (Hamada et al., 1996, Sandig et al., 1997) but delivery of functional protein into a sufficiently high percentage of surviving cells remains a difficulty.

Also known from US 5,484,710 (La Jolla: JC Reed et al) are regulatory elements linked to genes involved in cell death, as regulated by p53 tumour suppressor protein, and further proteins and their analogues for cell cycle control.

It remains desirable to provide particular further cell-delivery constructs for

useful proteins.

Summary and description of the invention

5 According to an aspect of the present invention, there are provided coupled proteins comprising transport protein sequences comprising sequences from herpesviral VP22 or from homologues or fragments thereof, together with sequences from other proteins selected from: (a) proteins for cell cycle control; (b) proteins that are conditionally cytotoxic or lethal upon administration (to a cell containing them) of
10 a corresponding drug, pro-drug or activator compound (otherwise described herein as suicide proteins); (c) antigenic sequences or antigenic proteins (e.g. of greater than 12 aminoacid residues in length) from microbial and viral antigens and tumour antigens; (d) immunomodulating proteins; and (e) therapeutic proteins. Examples of these kinds of proteins mentioned below. Thus, coupling or fusion to an aminoacid
15 sequence with the transport function of VP22 protein can provide a useful cell delivery construct for proteins of the kinds mentioned. (Where the context admits, 'coupling products' and similar expressions include reference to fusion proteins.)

 Preferably the coupled proteins are fusion proteins, which can conveniently be
20 expressed in known suitable host cells. Corresponding polynucleotide sequences can be prepared and manipulated using elements of per-se known and standard recombinant DNA technique and readily available adaptations thereof. However, chemically-coupled products can for certain applications be used if desired, and can be prepared from the individual protein components according to any of a variety of
25 per-se known chemical coupling techniques.

 VP22 or a functional sub-sequence thereof, optionally with an additional polypeptide tail for coupling, can be linked to other proteins or nucleic acid by chemical coupling in any known suitable standard manner.

30 Also provided by the invention are polynucleotides encoding the fusion proteins as described herein, including sequences corresponding to VP22 and another protein of one of the kinds mentioned above, and expression cassettes, plasmids, vectors and recombinant cells comprising the polynucleotides. These can be formed and used in
35 ways analogous to or readily adaptable from standard recombinant DNA technique.

Thus, corresponding polynucleotides can encode a fusion polypeptide that comprises a sequence with the transport function of herpesviral VP22 protein and a sequence with one of the functions specified herein. The polynucleotide can be comprised in an open reading frame operably linked to a suitable promoter sequence, and can
5 according to examples of the invention form part of an expression vector, e.g. comprising the polynucleotide carried in a plasmid. The expression vector can be for example a recombinant virus vector or a non-viral transfection vector. The vectors can for example be analogues or examples of those vectors mentioned or described in WO97/05265, or of those mentioned or described in WO 92/05263, WO 94/21807,
10 or WO 96/26267. For nucleotide sequence that are capable of being transcribed and translated to produce a functional polypeptide, degeneracy of the genetic code results in a number of nucleotide sequences that encode the same polypeptide. The invention includes all such sequences.

15 Thus products described herein can be used according to the invention as transportable proteins capable of being taken up by a target population of cells, e.g. so that an effector function corresponding to the polypeptide sequence coupled to the VP22, from among the kinds mentioned above, can take place within the target cells that have taken up the product. Thus, for example, the target cells may present
20 desired tumour antigen epitopes in a case where the polypeptide sequence is from a chosen tumour antigen, or become subject to cell cycle control effects where the the polypeptide sequence is from a cell cycle control protein, or become in some degree susceptible to cell killing or injury after additional treatment with a prodrug where the polypeptide sequence is from a corresponding 'suicide protein'. In use,
25 many of the products described herein can be expressed as fusion proteins in a first part of the target population of cells, exported therefrom, and taken up by a second part of the target population of cells not directly producing the protein. Also within the invention are mammalian and microbial host cells comprising such vectors or other polynucleotides encoding the fusion proteins, and their production and use.

30

A fusion polypeptide as described herein can be transported to a target population of cells, by introducing a polynucleotide or other vector encoding the fusion polypeptide into a first part of the target population of cells, e.g. by transfection or microinjection; expressing the encoding polynucleotide to produce the fusion
35 polypeptide, thereby to cause it to be exported from said first part of said target

population, and to cause it to be taken up by a second part of the target population of cells not directly producing the fusion polypeptide.

5 Coupling products (including chemically coupled products) can also be transported into a target population of cells by directly exposing the cells to a preparation of the coupling products, thereby to cause the target cells to take them up.

10 In this specification, 'VP22' denotes: protein VP22 of HSV, e.g. of HSV1, and transport-active fragments and homologues thereof, including transport-active homologues from other herpesviruses including varicella zoster virus VZV, equine herpesvirus EHV and bovine herpesvirus BHV; modified and mutant proteins and fusion polypeptides and coupling products having homology therewith and a transport function corresponding to a transport function of VP22 of HSV1; and in context also
15 relates to nucleic acid sequences encoding any of the above whether in the form of naked DNA or RNA or of a vector, or of larger nucleic acid sequences including such sequences as sub-sequences.

20 Among sub-sequences of herpesviral VP22 protein with transport activity we have found that for example transport activity is present in polypeptides corresponding to aminoacids 60-301 and 159-301 of the full HSV1 VP22 sequence (1-301). For the sequence, see e.g. Figure 4 in WO 97/05265. A polypeptide consisting of aa 175-301 of the VP22 sequence has markedly less transport activity, and is less preferred in connection with the present invention. Accordingly, the present invention relates in
25 one aspect to coupled and fusion proteins comprising a sub-sequence of VP22 containing a sequence starting preferably from about aa 159 (or earlier, towards the N-terminal, in the native VP22 sequence), to about aa301, and having (relative to the full VP22 sequence) at least one deletion of at least part of the VP22 sequence which can extend for example from the N-terminal to the cited starting point, e.g. a deletion
30 of all or part of the sequence of about aa 1-158. (Less preferably, such a deletion can extend further in the C-terminal direction, e.g. to about aa 175.) For example, partial sequences in the range from about aa 60-301 to about aa 159-301 are provided.

35 VP22 sequences as contemplated herein extend to homologous proteins and

fragments based on sequences of VP22 protein homologues from other herpesviruses, e.g. the invention provides corresponding derivatives and uses of the known VP22-homologue sequences from VZV (e.g. all or homologous parts of the sequence from aa 1-302), from MDV (e.g. all or homologous parts of the sequence from aa 1-249) and from BHV (e.g. all or homologous parts of the sequence from aa 1-258). The sequences of the corresponding proteins from HSV2, VZV, BHV and MDV are available in public protein/nucleic acid sequence databases. Thus, for example, within the EMBL/Genbank database, a VP22 sequence from HSV2 is available as gene item UL49 under accession no. Z86099 containing the complete genome of HSV2 strain HG52; the complete genome of VZV including the homologous gene/protein is available under accession numbers X04370, M14891, M16612; the corresponding protein sequence from BHV is available as 'bovine herpesvirus 1 virion tegument protein' under accession number U21137; and the corresponding sequence from MDV is available as gene item UL49 under accession number L10283 for 'gallid herpesvirus type 1 homologous sequence genes'. In these proteins, especially those from HSV2 and VZV, corresponding deletions can be made, e.g. of sequences homologous to aa 1-159 of VP22 from HSV1. These cited sequences are hereby incorporated herein by reference. Homologies between them are readily accessible by the use of standard algorithms and software, for example those mentioned in WO 95/12673, page 9.

Furthermore, chimeric VP22 proteins and protein sequences are also useful within the context of the present invention, e.g. a protein sequence from VP22 of HSV1 for part of which a homologous sequence from the corresponding VP22 homologue of another herpesvirus has been substituted. For example, into the sequence of polypeptide 159-301 from VP22 of HSV1, C-terminal sequences can be substituted from VP22 of HSV2 or from the VP22 homologue of BHV.

It has been found that deletion of the 34-aminoacid C-terminal sequence from VP22 of HSV1 abolishes transport-activity, thus this sequence region contains essential elements for transport activity. According to a further aspect of the invention, there are provided coupled and fusion polypeptides comprising the 34-aminoacid C-terminal sequence from VP22, or a variant thereof, together with a sequence from another protein selected from: (a) proteins for cell cycle control; (b) proteins that are conditionally cytotoxic or lethal upon administration (to a cell

containing them) of a corresponding drug or activator compound; (c) antigenic sequences or antigenic proteins (e.g. of greater than 12 aminoacid residues in length) from microbial and viral antigens and tumour antigens; (d) immunomodulating proteins; and (e) therapeutic proteins. These are provided for example for use by administration in the form of protein to cells that will take them up. Coupled products of modified terminal fragments having at least one mutation insertion or deletion relative to the C-terminal 34 aminoacid sequence of HSV1 VP22 are also provided.

It has also been found that sequences necessary for transport activity contain one or a plurality of aminoacid sequence motifs or their homologues from the C-terminal sequence of VP22 of HSV1 or other herpesviruses, which can be selected from RSASR, RTASR, RSRAR, RTRAR, ATATR, and wherein the third or fourth residue A can be duplicated, e.g. as in RSAASR. Corresponding fusion polypeptides with proteins of the kinds mentioned herein are also provided.

In addition to their uses as indicated elsewhere herein, the coupled and fusion polypeptides can also be used to raise antibodies which can be used in diagnostic and monitoring specific binding assays in per-se known manner, e.g for monitoring the intracellular localization of the coupled or fusion proteins themselves or their components.

('VP22' herein is not intended to include natural unmodified VP22 protein or corresponding gene in its natural and unmodified association with herpes virus in its various natural lifecycle stages, e.g. in association with herpesvirus which has not been subjected to genomic alteration. However, 'VP22' does for example refer to the corresponding protein or gene of a virus which has for example been altered in respect of its UL49/VP22 gene or function, or which has had inserted into its genome an additional and/or hybrid VP22 gene.)

The coupling products or fusion proteins based on VP22 can have a range of molecular sizes. The products can in practice be for example up to about 70kDa or more, e.g. 90kDa or 100kDa or more in respect of the size of the protein to be coupled or fused to VP22. The embodiments of the invention include examples where the fusion peptide is e.g. at least about 13 residues long, or more than about 12 aminoacid residues long, e.g. other than a 12-residue antigenic epitope peptide. The

proteins to be fused can sometimes also be more than about 27 or 32 kDa, e.g. they can be other than 27kDa in size. For example, one of the proteins that can be thus coupled, p53, itself has a size of about 53kDa. The coupled polypeptide or fusion protein, including the VP22 component can have a size up to about 120 kDa, e.g. up to about 80kDa or 100kDa.

It is sometimes preferred that the VP22 sequence is fused at its N-terminus to the sequence of the chosen other protein of one of the kinds mentioned herein. C-terminal fusions can sometimes be correspondingly less preferred.

10

In the polypeptides of the invention, mutations of the constituent aminoacid sequences (including those of the immunomodulatory and other proteins mentioned herein) can be incorporated in the fusion polypeptides and other coupled proteins. Included here are proteins having mutated sequences such that they remain homologous, e.g. in sequence, function, and antigenic character or other function, with a protein having the corresponding parent sequence. Such mutations can preferably for example be mutations involving conservative aminoacid changes, e.g. changes between aminoacids of broadly similar molecular properties. For example, interchanges within the aliphatic group alanine, valine, leucine and isoleucine can be considered as conservative. Sometimes substitution of glycine for one of these can also be considered conservative. Interchanges within the aliphatic group aspartate and glutamate can also be considered as conservative. Interchanges within the amide group asparagine and glutamine can also be considered as conservative. Interchanges within the hydroxy group serine and threonine can also be considered as conservative. Interchanges within the aromatic group phenylalanine, tyrosine and tryptophan can also be considered as conservative. Interchanges within the basic group lysine, arginine and histidine can also be considered conservative. Interchanges within the sulphur-containing group methionine and cysteine can also be considered conservative. Sometimes substitution within the group methionine and leucine can also be considered conservative. Preferred conservative substitution groups are aspartate-glutamate; asparagine-glutamine; valine-leucine-isoleucine; alanine-valine; phenylalanine-tyrosine; and lysine-arginine. In other respects, mutated sequences can comprise insertion and/or deletions. The mutated protein sequences can additionally or alternatively be encoded by polynucleotides that hybridize under stringent conditions with the appropriate strand of the naturally-occurring

polynucleotide encoding the parent protein, and can be tested for positive results in known functional tests relevant to the parent protein. ('Stringent conditions' are sequence dependent and will be different in different circumstances. Generally, stringent conditions can be selected to be about 5 deg C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and Ph) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typically, stringent conditions will be those in which the salt concentration is at least about 0.02 molar at pH 7 and the temperature is at least about 60 deg C. As other factors may affect the stringency of hybridization, including, among others, base composition and size of the complementary strands, the presence of organic solvents and the extent of base mismatching, the combination of parameters is more important than the absolute measure of any one.)

15 Coupling with cell cycle control proteins:

In one useful class of embodiments of the invention, VP22 can be coupled with per-se known cell cycle control proteins. Thus, in an example of the invention concerned with cell cycle control, as particularly described in an example below, VP22 can be coupled with p53 protein. A purpose and use here can be to block cell cycle progression, especially in malignant cells.

VP22 can also usefully be coupled with cyclin-dependent kinase inhibitors, e.g. p16, p21 or p27. Normal cell cycle progression requires these proteins; absence of these can derepress the cell cycle, and corresponding coupling products can be used for treatment of cancer cells.

VP22 coupling products can also usefully be used in the modulation of apoptosis, e.g. to induce cell death, of the apoptosis type, by the introduction into a cell of a protein apoptotic domain coupled to VP22, such as e.g. apoptosis protein bax, or its known identified apoptotic inducing peptide; or known related protein bad or bak. Here too the coupling product can be applied in the form either of protein or DNA encoding it. VP22 coupling products can be used in the form of VP22 with known proteins of the bcl2 family, such as bcl2 itself, bcl-xL, or bclw, to mask or inhibit apoptosis where this is desired, e.g. in treatment of neurodegeneration.

Other VP22 coupling products can be used to promote apoptosis, comprising VP22 linked with known ICE-like proteases. VP22 linkage products with inhibitors of ICE-like proteases, eg pseudosubstrates, can be used to mask or overcome the apoptosis-stimulating effects the proteases themselves.

5

Thus, according to an embodiment of the invention there is provided a fusion polypeptide comprising an aminoacid sequence with the transport function of herpesviral VP22 protein and a sequence with the cell cycle control functionality of p53 protein. The fusion polypeptide can include for example substantially the full length p53 sequence or substantially the full length VP22 sequence, or both.

10

Fusion with VP22 can thus be used for delivery of an agent for cell cycle control such as p53. (Where the description given herein refers to p53 and related peptides, it will be understood that, where the context admits, alternative cell cycle control agents, such as for example those p53 analogues and other cell cycle control proteins mentioned and referred to herein, are also contemplated, as are, more generally, alternative fusion or coupling partners for VP22, of any of the other types mentioned herein.) Once expressed in a subpopulation of expressing cells, such a fusion protein can be transported by the VP22 transport mechanism from the expressing cell into a significant proportion of surrounding cells, and the foreign attached polypeptide can then exert its functionality.

15

20

Also provided by this aspect of the invention are corresponding polynucleotides, encoding a fusion polypeptide that comprises a sequence with the transport function of herpesviral VP22 protein and a sequence with the human/mammalian cell cycle-regulating function of p53 protein. The polynucleotide can be comprised in an open reading frame operably linked to a suitable promoter sequence.

25

30

The polynucleotide can according to examples of the invention form part of an expression vector, e.g. comprising the polynucleotide carried in a plasmid. The expression vector can be for example a virus vector or a non-viral transfection vector. The vectors can for example be analogues or examples of those described and referred to in WO 97/05265 or Elliott and O'Hare (1997).

35

Also provided by the invention are methods of inhibiting cell division, which comprise exposing cells that have insufficient active/free p53 to arrest their cell cycle, to contact with a fusion polypeptide as described herein.

5 Among the methods of the invention is a method of inhibiting tumour cell division, which comprises exposing a tumour cell present in a tumour cell mass, the tumour cell comprising insufficient active/free p53 to arrest its cell cycle, to contact with a vector as described herein, thereby causing the cell to express a fusion polypeptide as described herein and to expose other cells of the tumour cell mass to
10 contact with the fusion polypeptide.

 We have shown (see description below) that VP22-p53 can be transported to many untransfected cells in a monolayer. The fusion protein can be functional in cell cycle arrest and/or induction of apoptosis, for example both in primary expressing
15 cells and in cells which have received VP22 via cell-to-cell spread. For example, the fusion protein can be applied to a p53 negative osteosarcoma cell line SAOS-2 (Diller et al., 1990). Functional p53 expressed in these cells causes cell cycle arrest at the G₁-S boundary and ultimately cell death, this can be assayed using confocal microscopy and antibodies against specific cell cycle markers. Function of the p53
20 fusion protein can also be used and assessed in other tumorigenic cell lines where p53 is present but contains specific and well characterized point mutations leading to non-functionality.

 A number of vector systems such as retroviral or adenoviral infection or the
25 injection of protein-liposome complexes can be readily adapted to form examples of this invention for the administration of cell-cycle control proteins to cells and tissues of human and non-human animal subjects to be treated. For example, in relation to work on p53 protein alone, these have clearly demonstrated that addition of wild type p53 protein can curtail cancerous cell growth in vivo. A number of therapeutic
30 applications of non-invasive delivery of VP22 coupling products with cell-cycle control proteins will be apparent to the skilled reader.

 For example, naked DNA for a VP22-protein fusion with a tumour effector protein such as p53 can be injected into a tumour, e.g. a solid tumour, e.g. a solid
35 tumor selected by molecular diagnostics for lack of functional p53.

Recombinant viruses can be used as mentioned, encoding and able to express VP22-p53 and equivalently-functioning fusion proteins. For example an adenovirus can express VP22-p53 and can be made dependent on a tumour-specific promoter to drive an essential viral gene such as E1a. More generally, a recombinant virus vector carrying such a fusion can be defective, non-replicating or replication-restricted so that replication is dependent on conditions prevailing in the target tissue or cell but not in normal or non-target cells.

In certain examples of the invention, the protein having p53 functionality can for example comprise variants or mutants of p53, for example those variants as described in specification WO 97/04092 (Rhone Poulenc Rorer SA: Bracco L, Conseiller E) ("New p53 variants e.g. with oligomerisation domain replaced by leucine zipper - useful for treating hyper-proliferative disorders, especially cancer and restenosis"), which describes inter alia the following variant proteins: (a) variants of protein p53 having at least part of the oligomerisation domain deleted and replaced by a leucine zipper domain; (b) variants of p53 preferentially active in transformed cells, where all or part of at least one functional domain has been deleted and replaced by a heterologous domain preferentially active in such cells; (c) variants of p53 with a deletion in the C-terminal part, from residue 366, followed by a 19 amino acid sequence (encoded by a 76 bp fragment reproduced in the specification) representing the last part of the alternatively spliced part of murine p53; and (d) chimeric protein containing a transactivating domain, a DNA-binding domain, a nuclear localisation domain and an oligomerisation domain, in which DNA-binding domain and the nuclear localisation domain comprise amino acids 75-325 or 75-336 of human wild-type p53.

In further examples of the invention, vectors and fusion proteins can encode or comprise variant p53 polypeptides comprising chimaeric p53 sequences including heterologous tetramerisation domains, which can be adapted from those described in specifications WO 96/16989 and US 5,573,925 (Wistar Institute of Anatomy & Biology: Halazonetis TD) and used in corresponding ways. In such examples of the invention, the p53 sequences can comprise chimaeric p53 protein having a native p53 sequence and a heterologous tetramerisation domain that forms homotetramers such that the resulting chimaeric protein cannot hetero-oligomerise with wild-type or tumour

derived mutant p53 and does not interfere with the native p53 tumour suppressing functionality.

5 Fusion proteins and vectors according to further examples of the present invention can be used for treatment of hyperproliferative disease, especially cancer and autoimmune disease, e.g. restenosis, and particularly for treatment of cells having a p53 mutation and which also express protein MDM2 at high level, including for example HPV-related cancer cells. They may also be used to kill hyperproliferating cells in vitro. Such variants can involve active and stable tumour suppressors and
10 apoptosis-inducing agents and are proposed to be active where the wild type protein is not, i.e. not inactivated by dominant negative or oncogenic mutants, nor by other cellular proteins (because the leucine zipper domain prevents formation of inactive mixed oligomers).

15 Fusion proteins and vectors can also be used, according to further examples of the present invention, in medicaments for suppressing neoplastic phenotype of cancer cells lacking wild-type p53 protein, in ways e.g. corresponding to the use of wild-type p35 gene as described in specification EP 0 710 722 (Univ California: Chen P, Lee W), which describes genes and retroviral vectors for the purposes inter alia of
20 suppressing neoplastic phenotype in cancer cells such as osteosarcoma cells, lung carcinoma cells, colon carcinoma cells, lymphoma cells, leukaemia cells, soft tissue sarcoma cells or breast, bladder or prostate carcinoma cells.

25 Fusion proteins and vectors can also be used according to further examples of the present invention, e.g. in ways corresponding to those described in specification WO 95/12660 (Univ Texas System: Roth JA et al), which describes recombinant adenovirus which carries an adenovirus vector construct comprising an expression region encoding p53, and which is capable of expressing the p53 in for example human malignant cells, and which can be used inter alia for regional delivery of
30 tumour suppressor gene p53, to diseased cells, either to restore p53 function to p53 deficient cells, or to suppress tumour growth in cells having abnormal p53, and thus

to treat human malignancies such as breast and lung cancer. Such adenovirus may also be used for in vitro analyses and mutagenesis studies of various p53 genes.

5 Fusion proteins and vectors can also be used, according to further examples of the present invention as inhibitors of hepatitis B virus (HBV) replication, in ways corresponding to those described in US 5,635,473 and WO 96/11017 (Mogam Biotechnology Research Institute: H-S Lee et al).

10 Screening assays for identifying agents that effectively increase the level of cell death, and which can act as p53 analogues and can induce apoptosis in cells, are described for example in US 5,484,710 (La Jolla: JC Reed et al), particularly in example IV thereof. Also contemplated as alternative embodiments of the invention are fusion proteins and related materials incorporating VP22 functionality and Bax protein functionality. In relation to Bax protein, reference is made to US 5,484,710
15 and references cited therein, incorporated herein by reference.

Coupling with 'suicide protein':

In a further class of embodiments of the invention, VP22 or a functional sub-
sequence thereof can be usefully coupled or fused with for example a 'suicide protein'
20 such as for example the known thymidine kinase, nitroreductase, or other enzyme or functional fragment thereof known as applicable for a similar purpose. The coupling product can penetrate into cells which are to be treated with (in the case of thymidine kinase) ganciclovir or another drug (prodrug) of the same family, so that the prodrug is converted in the cells containing the 'suicide gene' product to an active form to kill
25 the cells.

Suitable examples of useful known suicide genes and corresponding pro-drugs are given and referred to for example in WO 94/13824 (Univ Curie Paris: M Caruso et al), in WO 95/05835 (Baylor College: S Chen et al), and in WO 93/08288 (Cancer

Research Campaign Technology: G Anzelark et al), and WO 93/01281 (US DHHS: RM Blaese et al), and include, besides thymidine kinase (suicide gene) and ganciclovir/acyclovir (prodrug), nitroreductase (suicide gene) and CB1954 (prodrug), and cytosine deaminase (suicide gene) and 5-fluorocytosine (prodrug). These and
5 other suicide proteins and corresponding (pro)drugs are also reviewed and their uses mentioned in 'Genetic Prodrug Activation Therapy', A Rigg and K Sikora, Molecular Medicine Today, Aug 1997, pp 359-366.

Where the VP22-TK fusion is presented in the form of DNA in any of the ways
10 described in WO 97/05265 or Elliott and O'Hare (1997), a target cell can be transfected with the gene encoding this fusion, and the expressed fusion can then be translocated out of the cell in which it was expressed and into surrounding cells - producing a killing effect on such cells when treated with ganciclovir etc, an effect which is different from, and can be additional to, known bystander effects.
15 Alternatively, as with other embodiments, such a VP22-TK fusion can be applied directly as protein.

Coupling with antigens:

In further embodiments, the invention concerns for example transport proteins
20 related to VP22 or its active fragments fused in fusion polypeptides or otherwise coupled with antigenic sequences or proteins (e.g. of greater than 12 aminoacid residues in length) selected for example from any of the antigenic materials or other proteins and peptides mentioned below.

25 In addition to the fusion polypeptides and coupling products, the invention provides coupling hybrids comprising VP22 coupled to a DNA that can for example comprise suitable known regulatory elements so that it can be transcribed and translated, and containing an open reading frame encoding any of the proteins mentioned below.

30

Coupling with antigens: VP22 can usefully be coupled with examples of microbial and viral antigens and of tumour antigens such as those mentioned below.

5 Treatment with coupling products of VP22 involving antigens of pathogens as provided hereby can evoke useful immune response against corresponding pathogens. Examples of such antigens are papilloma virus proteins L1 and L2. HIV proteins, gag, pol, env and nef, chlamydia antigens (such as the chlamydia Major Outer Membrane Protein MOMP) and Chlamydia heat shock proteins.

10 VP22 can also usefully be coupled with antigens from mycobacteria such as antigen from *Mycobacterium tuberculosis*.

Alternatively the antigen can be a tumour associated antigen, whereby the anti- tumour activity of the CTLs associated with tumour cell depletion is enhanced.

15 It has been found that specific cytokines such as tumour necrosis factor- α , interferon gamma, interleukin-2, interleukin-4 and interleukin-7 are particularly useful in this regard. Tumour associated antigens and their role in the immunobiology of certain cancers is discussed for example by P van der Bruggen et al., Current Opinion in Immunology, 4(5) (1992) 608-612. Particular examples of such antigens which are

20 envisaged for use in the context of the present application are E6 and E7 antigens of human papillomavirus (especially for example of types 6, 11, 16, 18, etc); Epstein-Barr virus-derived proteins, e.g. those identified in references 24 and 25 in P van der Bruggen et al., cited above: antigens of the MAGE series as identified in T. Boon, Adv Cancer Res 58 (1992) pp 177-210 and/or MZ2-E and other antigens as identified in

25 P. van der Bruggen et al, Science 254 (1991) 1643- 1647; melanoma proteins, e.g. human tyrosinase: and mucins such as those identified in P.O. Livingston, in Current Opinion in Immunology 4 (5) (1992) pp 624-629: e.g. MUC1 as identified in J Burchell et al, Int J Cancer 44 (1989) pp 691-696.

30 VP22 can also be usefully coupled with viral proteins such as glycoprotein

antigens, e.g. from herpesviruses, such as gH or gD or gB of herpes simplex virus; or gp50 of pseudorabies virus, as an example of an antigen of a veterinary pathogen, in this case a veterinary virus.

5 VP22 thus can be usefully coupled with antigens known from the prior art of malignant tumour treatment, including studies that have highlighted the potential for therapeutic vaccination against tumours using autologous material derived from a patient's own tumour. The theory behind this approach is that tumour cells may express one or more proteins or other biological macromolecules that are distinct from
10 normal healthy cells, and which might therefore be used to target an immune response to recognise and destroy the tumour cells.

 These tumour targets may be present ubiquitously in tumours of a certain type. A good example of this in cervical cancer, where the great majority of tumours
15 express the human papillomavirus E6 and E7 proteins. In this case the tumour target is not a self protein, and hence its potential as a unique tumour-specific marker for cancer immunotherapy is clear.

 There is increasing evidence that certain self proteins can also be used as
20 tumour target antigens. This is based on the observation that they are expressed consistently in tumour cells, but not in normal healthy cells. Examples of these include the MAGE family of proteins. It is expected that more self proteins useful as tumour targets remain to be identified.

25 Tumour associated antigens and their role in the immunobiology of certain cancers are discussed for example by P van der Bruggen et al, in Current Opinion in Immunology, 4(5) (1992) 608-612. Other such antigens, of the MAGE series, are identified in T. Boon, Adv Cancer Res 58 (1992) pp177-210, and MZ2-E and other related tumour antigens are identified in P. van der Bruggen et al, Science 254 (1991)
30 1643-1647; tumour-associated mucins are mentioned in P0 Livingston, in Current

Opinion in Immunology 4 (5) (1992) pp 624-629: e.g. MUC1 as mentioned in J Burchell et al, Int J Cancer 44 (1989) pp 691-696.

Coupling with immunomodulating proteins:

5 Embodiments of the invention of use in immune modulation include for example the following. VP22 can usefully be coupled with examples of cytokines or of other immunomodulatory compounds as mentioned below. Thus, VP22 can also be usefully coupled with immuno modulating proteins, e.g. those which enhance the immune response including the cytokine, interleukin 1, interleukin 2 and granulocyte-
10 macrophage colony stimulating factor (GM-CSF). Such products can for example be used in ways analogous to those mentioned in for example WO 96/26267 or WO 97/14808, to alter, e.g. to increase, an immune reponse specific to a target cell type, e.g. a tumour cell type, which has been exposed to the product either in-vitro or in-vivo.

15

 As used herein, the expression "immunomodulatory protein" and related terms includes a protein or proteins which either enhance or suppress a host immune response to a mutant virus or protein encoded thereby, or to an antigen such as an immunogen from a pathogen or source exogenous to the virus, or a tumour-
20 associated antigen. The immunomodulating proteins are not normally those proteins presently used as immunogens (antigens) in themselves. An immunomodulatory protein can be a natural member of a human or non human animal immune system, e.g. of a mammalian immune system, with a functional binding capacity for another natural constituent of such an immune system. Alternatively an immunomodulatory
25 protein can be a protein encoded by a pathogen, which has a functional binding capacity for a natural constituent of such an immune system.

 Alternatively an immunomodulatory protein can be an artificial protein, for example a fragment of a natural immunomodulatory protein, or a mutein of such a
30 protein or fragment, or a fusion protein incorporating any of these. Many immunomodulatory proteins, and genetic materials encoding them, and their

nucleotide and aminoacid sequences, are known to the literature of this subject, and available in genetic sequence databases such as the EMBL database, and several are commercially available in the form of engineered genetic material for cloning and other manipulation.

5

Immunomodulating proteins coupled with VP22 as described herein can usefully for example be of sequences native to the species which is to receive treatment with these coupling products or with DNA e.g. in the form of recombinant viruses, e.g. an immunomodulating protein of human type for treatment of a human subject.

10

Examples of useful known immunomodulating proteins in this connection include cytokines, chemokines, complement components, immune system accessory and adhesion molecules and their receptors of human or non-human animal specificity. Useful examples include GM-CSF, IL-2, IL-12, lymphotactin, CD40, and CD40L. Further useful examples include interleukins for example interleukins 1 to 15, interferons alpha, beta or gamma, tumour necrosis factor, granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), chemokines such as neutrophil activating protein (NAP), macrophage chemoattractant and activating factor (MCAF), RANTES, macrophage inflammatory peptides MIP-1 and MIP-1b, complement components and their receptors, or an accessory molecule such as B7.1, B7.2, ICAM-1, 2 or 3 and cytokine receptors.

15

20

25

OX40 and OX40-ligand (OX4OL) (gp34) (see e.g. WO 95/12673, WO 95/21251 and WO 21915) are further useful examples of immunomodulatory proteins. Immunomodulatory proteins can for various purposes be of human or non-human animal specificity and can be represented for present purposes, as the case may be and as may be convenient, by extracellular domains and other fragments with the binding activity of the naturally occurring proteins, and muteins thereof, and their fusion proteins with other polypeptide sequences, e.g. with immunoglobulin heavy

30

chain constant domains. Where nucleotide sequences encoding more than one immunomodulating protein are inserted, they can for example comprise more than one cytokine or a combination of cytokine(s) and accessory/adhesion molecule(s).

5 Immune response evoked by the use of such VP22 coupling products or by vectors encoding them can include immune responses of a variety of types, e.g. a response against a virally-encoded protein, and/or a response against a host antigen, being a response stimulated by a viral vector or by the expression of a heterologous gene encoded thereby, e.g. the coupling product with VP22. Among the uses of the
10 mutant virus vectors as described herein is e.g. to protect a subject of a susceptible species against infection by a corresponding wild-type virus when the subject is treated therewith, e.g. infected therewith, e.g. by direct immunisation.

 An immunomodulatory protein to be coupled with VP22 can be itself a hybrid
15 or fusion protein which comprises a polypeptide region having homology to and functionality of an immunomodulatory protein, linked to a polypeptide region having another homology and optionally another functionality. For example, the immunomodulatory protein can be, comprise, or correspond in functionality to the gp34 protein identified as a binding partner to human Ox-40 (see W Godfrey et al, J
20 Exp Med 180(2) 1994 pp 757-762, and references cited therein, including S Miura et al, Mol Cell Biol 11(3) 1991, pp 1313-1325). The version of this protein functionality that can be encoded in the mutant viral genome can correspond to the natural gp34 sequence itself, or to a fragment thereof, or to a hybrid expression product e.g. based on the (C terminal) extracellular (binding) domain of gp34 fused to another protein,
25 e.g. to the constant region of an immunoglobulin heavy chain such as human IgG1, e.g. with the extracellular domain of gp34 (a type 2 membrane protein) fused at its N-terminal to the C-terminal of the immunoglobulin constant domain.

 Others of the immunomodulatory proteins can also be carried and expressed
30 in such derivative and hybrid forms, including mutated forms as mentioned herein.

In certain examples the immunomodulating protein can comprise a cytokine, preferably granulocyte macrophage colony stimulating factor (GM-CSF), e.g. murine or preferably human GM-CSF.

5 Murine and human GM-CSFs are both known: the murine GM-CSF gene encodes a polypeptide of 141 amino acids, the mature secreted glycoprotein having a molecular weight of between 14k-30k daltons depending on the degree of glycosylation. GM-CSF generically is a member of the haematopoietic growth factor family and was first defined and identified by its ability to stimulate in vitro colony
10 formation in haematopoietic progenitors. GM-CSF is a potent activator of neutrophils, eosinophils and macrophage-monocyte function, enhancing migration, phagocytosis, major histocompatibility complex (MHC) expression, and initiating a cascade of bioactive molecules which further stimulate the immune system. GM-CSF is currently being clinically evaluated for treatment of neutropenia following chemotherapy and as
15 an adjuvant in cancer therapy.

The heterologous nucleotide sequence employed can comprise a heterologous gene, gene fragment or combination of genes provided it encodes an immunomodulating protein as defined above.

20

According to examples of the invention, combinations of two or more immunomodulatory proteins can be used for the purposes described herein. In particular examples, given for illustration only and not limitation, combinations involving IL2, GMCSF, lymphotactin and/or CD4OL can be used with each other or
25 with others of the immunomodulatory proteins cited above. Each of the other binary combinations of the immunomodulatory proteins mentioned above are also given by, and within the scope of, this disclosure.

Other coupling products:

In certain embodiments the invention can be useful in gene therapy applications: thus, for example, VP22 can also be usefully coupled with examples of genes used or proposed to be used in gene therapy including: the gene for human adenosine deaminase (ADA), as mentioned in for example WO 92/10564 (KW Culver et al: US Secretary for Commerce & Celco Inc), WO 89/12109 & EP 0 420 911 (IH Pastan et al): the cystic fibrosis gene and variants described in WO 91/02796 (L-C Tsui et al: HSC Research & University of Michigan), in WO 92/05273 (FS Collins & JM Wilson: University of Michigan) and in WO 94/12649 (RJ Gregory et al: Genzyme Corp).

VP22 can also usefully be coupled with known transcriptional regulatory proteins such as NF-AT, which becomes activated by translocation to the nucleus and induces transcription of interleukin e.g. of IL1. The coupling with VP22 can be used here to avoid retention of the coupled product in the cytoplasm.

The invention also includes coupled and fusion proteins in which a linker sequence is provided that enables the fusion protein to be split intracellularly to enable separation of the antigenic part, such as that mentioned above, from the transport protein part. A cleavage-inducing sequence can comprise for example the aminoacid sub-sequence RVCSNPPCETHETGTTNTATATSN or other cleavage sequences indicated for example in AC Wilson et al, in Genes and Development 9 (1995) 2445-2458.

Also provided by the present invention are processes for treating cells with coupling products as described herein, so as to produce immunogenic, immunomodulatory, cytotoxic/lethal or therapeutic effects.

Examples of materials and processes as described herein are useful in the modulation of cellular activity, e.g. with the aim and effect of producing or altering immune responses, for example for the prophylaxis or therapy of disease, e.g. the

production of immune responses against pathogens or tumours.

Other uses for certain of the materials and processes hereof include the regulation of gene expression in cells, e.g. for purposes of corrective gene therapy
5 and/or for reducing or controlling tumour cell growth and activity. Cell treatments according to the invention can be in-vitro, ex-vivo or in-vivo.

Among the derivatives of VP22 that can be used according to aspects of the invention as transport active substances and for coupling with materials to be
10 transported, for the purposes set forth elsewhere herein, are peptides comprising a transport-active functional sequence from the C terminal section of VP22.

Non-limitative examples of treatment methods using the materials described herein comprise treatment of antigen-presenting cells or cell preparations containing
15 them with a fusion of VP22 and an antigen, e.g. one of those antigens mentioned above, (or with a vector, e.g. a viral vector, encoding such a fusion), so as to procure processing of the antigen and presentation by the MHC I route so as to procure a CTL response to the antigen. The methods so provided include priming and expansion of T cells and adoptive immunotherapy using the materials so obtained, in a manner
20 otherwise analogous to known priming, expansion and adoptive immunotherapy methods.

A number of vector systems such as retroviral or adenoviral infection or the injection of protein-liposome complexes, as well as herpesviral vector systems, can
25 be readily adapted to form examples of this invention. For example, naked DNA for a VP22-protein fusion with a protein of one of the kinds mentioned herein can be injected into a tissue to be treated, according to the nature and purpose of the protein to be delivered. Recombinant viruses can be used as mentioned, encoding and able to express VP22 fusion proteins. A recombinant virus vector carrying such a fusion
30 can be defective, non-replicating or replication-restricted so that replication is

dependent on conditions prevailing in the target tissue or cell but not in normal or non-target cells.

5 Vectors and fusion proteins of examples of the invention can be useful in gene therapy, and to treat or protect against abnormal cell proliferation, esp. cancer but also psoriasis, atherosclerosis and arterial restenosis, and to induce apoptosis of e.g. proliferating lymphocytes, i.e. to induce tolerance, e.g. to prevent transplant rejection or for treatment of autoimmune diseases such as systemic lupus erythematosus or rheumatoid arthritis.

10

In addition to medical therapeutic applications, the effect shown herein can also be exploited by assays, provided by the invention, which rely on substrate-enzyme interactions or the interaction of proteins expressed in different cellular populations.

15

An embodiment of the invention is further described, without intent to limit the invention, with reference to the accompanying drawings and to the materials and methods described below:

20

In the accompanying drawings,

--- Figure 1 illustrates that:

25

Mock-transfected cos-1 cells were labelled by indirect immunofluorescence with antibodies for VP22 (Figure 1a), p53 (Figure 1c) and the CMV epitope (Figure 1d) to establish the levels of background label. Cells transfected with pc49epB (Figure 1b) and labelled for VP22 demonstrate a typical VP22 cytoplasmic pattern with clear spread to the nuclei of adjacent cells. Cells transfected with the VP22-p53 fusion protein construct p4953ep+10 were labelled for VP22 and p53 (Figures 1e and

1f) or VP22 and epitope (Figures 1g and 1h): the fusion protein can be detected in the nuclei of cells adjacent to the primary expressing cell.

— Figure 2 is a plasmid map to illustrate p4953ep+10, encoding a fusion protein comprising sequences VP22, p53 and an epitope tag.

— Figure 3 illustrates that

Protein extracts from cos-1 cells transfected with a range of plasmid constructs were analysed by western blot. The panel shown leftmost has been probed with an antibody against VP22 and demonstrates that pUL49epB and pc49epB plasmids encoding VP22 alone generate a protein of 38kDa, the VP22-p53 fusion protein expressed from p4953ep+10 produces a protein of approx. 90kDa with very little degradation.

The panel shown rightmost has been probed with an antibody against p53 and demonstrates that cells transfected with plasmids encoding either p53 alone (pcB6+p53) or the p4953ep+10 fusion protein construct produce p53 protein at 53kDa. The p4953ep+10 construct also synthesises the VP22-p53 fusion protein at 90kDa, the p53 in this sample may be a degradation product or more likely endogenously induced p53.

Materials and Methods

Cell culture and transfection

Cos-1 cells were grown in Dulbecco's modified MEM supplemented with 10% new born calf serum at 37°C with 5% CO₂.

Transfections were performed using the BES/CaCl₂ method (Elliott and O'Hare, 1997) with 200ng test plasmid with 1800ng pUB19. Transfections were allowed to proceed for 48h at which point the monolayers were harvested for immunofluorescence or western blot analysis.

Immunofluorescence and antibodies

Cell monolayers on coverslips were fixed with 100% methanol for 15 mins at room temperature and labelled as described in Elliott and O'Hare (1997). All antibodies were diluted in PBS + 10% serum. VP22 was detected using a rabbit polyclonal antibody AGV30 (1:500), p53 was detected using a mouse monoclonal antibody DO-1 (Santa-Cruz Ltd), the CMV epitope was detected using a mouse monoclonal antibody CMV LNA (Capricorn Ltd). Images were obtained using a Bio-Rad MRC600 confocal microscope.

10 Plasmid Constructs

The VP22-p53 fusion protein construct was generated by cloning a full length p53 PCR fragment C-terminal to VP22 into a unique Bam site, keeping both VP22 and the CMV epitope in frame.

15 Western blot analysis

Western blots were probed with anti-VP22 (1:10,000), anti-p53 (1:1000).

We constructed an epitope-tagged full length in-frame VP22-p53 fusion protein construct (Fig 2). This vector generates a fusion protein of approx 90kDa when expressed in Cos-1 cells, with very little protein degradation as judged by western blot analysis (Fig 3). When tested for delivery by intercellular trafficking, the fusion protein appears to function exactly as VP22 alone. It is located in the cytoplasm of primary transfected cells as shown by immunofluorescence using methanol-fixed Cos-1 cell monolayers labelled with anti-VP22 (Figs 1e and 1g), -p53 (Fig 1f) or -epitope (Fig 1h) antibodies and is able to move very efficiently into the nuclei of neighbouring cells. The relative efficiency of transport has not been empirically determined but appears only slightly less than VP22 alone.

In further experiments, p53-negative osteosarcoma cells were transfected (using the calcium phosphate technique) with naked DNA expressibly encoding either

(a) wild-type VP22, (b) wild-type p53) or (c) the VP22-p53 fusion protein described above. The transfected cells (b) and (c) showed ability to undergo apoptosis, unlike the control cells (a), indicating that the VP22-p53 fusion protein retains the functionality of p53.

- 5 In variants of the example given here, VP22 deletion constructs with decreased fusion protein size can be made if desired, e.g. to improve rate or extent of transport, and without loss of protein function.

 In further variants, the order of the components of the fusion can be varied, e.g. the p53 and VP22 sequences can readily be included in the order opposite to the
10 order involved in the plasmid shown in Figure 2, with satisfactory results.

 The present disclosure extends to modifications and variations of the description given herein inclusive of the attached claims that will be apparent to the reader skilled in the art. The disclosure hereof, incorporating WO 97/05265 and of
15 Elliott and O'Hare (1997) which are made an integral part hereof, is intended to extend in particular to classes and subclasses of the products and generally to combinations and to subcombinations of the features mentioned, described and referred to in the disclosure. Documents cited herein, including the references below, are hereby incorporated in their entirety by reference for all purposes.

20

Additional References

 Diller, L., Kassel, J. Nelson, C.E., Gryka, M.A., Litwak, G., Gebhardt, M. Bressac, B., Ozturk, M., Baker, S.J., Vogelstein, B. and S.H. Friend, (1990) p53 functions as a cell cycle control protein in osteosarcomas. Mol. Cell. Bio. 10:5772-5781.

- 25 Elliott G. and P. O'Hare (1997) Intercellular trafficking and protein delivery by a herpesvirus structural protein. Cell 88:223-233.

 Ensoli, B., Buonaguro, L., Barillari, G., Fiorelli, V., Gendelman, R., Morgan, R.A., Wingfield, P. and R. C. Gallo. (1993) Release, uptake and effects of extracellular human immunodeficiency virus Tat protein on cell growth and viral transactivation. J.

Virol. 67:277-287.

Fawell, S., Seery, J., Daikh, Y., Moore, C., Chen, L.L., Pepinsky, B. and J. Barsoum. (1994) Tat-mediated delivery of heterologous proteins into cells. *Proc. Natl. Acad. Sci.* 91:664-668.

- 5 Hamada, K., Alemany, R., Zhang, W-W, Hittelman, W.N., Lotan, R., Roth, J.A. and M.F. Mitchell. (1996) Adenovirus-mediated transfer of a wild-type p53 gene and induction of apoptosis in cervical cancer. *Cancer Research* 56:3047-3054.

- Jackson, A., Friedman, S., Zhan, X., Engleka, K.A., Forough R. and T. Maciag. (1992) Heat shock induces the release of fibroblast growth factor 1 from NIH3T3 cells.
10 *Proc. Natl. Acad. Sci.* 89:10691-10695.

Kumar, X, M., Srinivas, S., Detolla, L.J., Yu S.F., Stass, S.A. and A.J. Mixson. (1997) Parenteral gene therapy with p53 inhibits human breast cancer tumors in vivo through a bystander effect without evidence of toxicity. *Hum Gene Therapy* 8:177-185.

- 15 Levine, A.J. (1997) p53, the cellular gatekeeper for growth and division. *Cell* 88:323-331.

Nielsen, L.L., Dell, J., Maxwell, E., Armstrong, L., Maneval, D. and J.J. Catino. (1997) Efficacy of p53 adenovirus-mediated gene therapy against human breast cancer xenografts. *Cancer Gene Therapy* 4:129-138.

- 20 Sandig, V., Brand, K., Herwig, S., Lukas, J., Bartek, J. and M. Strauss. (1997) Adenovirally transferred p16 and p53 genes cooperate to induce apoptotic tumor cell death. *Nature Med.* 3:313-319.

- Zheng, P.S., Iwasaka, T., Ouchida, M., Fukuda, K., Yokoyama, M. and H. Sugimori. (1996) Growth suppression of a cervical cancer cell line (TMCC-1) by the
25 human wild type p53 gene. *Gynecol Oncol.* 60:245-250.

CLAIMS:

- 1: Coupled polypeptides and fusion polypeptides comprising (i) an aminoacid sequence with the transport function of herpesviral VP22 protein and (ii) another
5 protein sequence selected from (a) proteins for cell cycle control; (b) suicide proteins (proteins that are conditionally cytotoxic or lethal upon administration, to a cell containing them, of a corresponding (pro)drug or activator compound); (c) antigenic sequences or antigenic proteins (e.g. of greater than 12 aminoacid residues in length) from microbial and viral antigens and tumour antigens; (d) immunomodulating
10 proteins; and (e) therapeutic proteins.
- 2: A polypeptide according to claim 1 where said other protein sequence is from a mammalian (e.g. human) cell cycle control protein.
- 15 3: A polypeptide according to claim 2 where said other protein sequence is from a mammalian (e.g. human) protein for increasing or inducing cell apoptosis or for conferring on a cell the ability to undergo apoptosis.
- 20 4: A polypeptide according to claim 2 where said other protein sequence is from a mammalian (e.g. human) cell cycle control protein selected from p53 protein, cyclin dependent kinase inhibitors, and proteins of the bcl2 and bax families.
- 5: A polypeptide according to claim 4, which is a fusion polypeptides and comprises a sequence from a p53 protein.
- 25 6: A fusion polypeptide according to claim 5, comprising substantially a full length VP22 sequence and substantially a full length p53 sequence.

- 7: A polypeptide according to claim 1 where said other protein sequence is from a suicide protein.
- 8: A polypeptide according to claim 7 where said suicide protein is selected from thymidine kinase and nitroreductase.
- 9: A polypeptide according to claim 7, comprising substantially the full length VP22 sequence.
- 10: A polypeptide according to claim 1, which is a fusion polypeptide and comprises a cleavage-inducing linker sequence located between the VP22 sequence and said other protein sequence.
- 11: A polypeptide according to claim 1, comprising a sub-sequence of HSV VP22 starting from about aa 159 and extending to about aa 301, and having (relative to the full VP22 sequence) at least one deletion of at least part of the VP22 sequence extending for example from the N-terminal to the sequence of about aa 1-158.
- 12: A polypeptide according to claim 1, which comprises a sequence corresponding to aminoacids 60-301 or 159-301 of the full HSV VP22 sequence.
- 13: A polynucleotide encoding a fusion polypeptide that comprises an aminoacid sequence with the transport function of herpesviral VP22 protein and a sequence from another protein selected from (a) antigenic sequences or antigenic proteins from microbial and viral antigens and tumour antigens; (b) immunomodulating proteins; (c) proteins that are conditionally cytotoxic or lethal upon administration (to a cell containing them) of a corresponding drug or activator compound; (d) proteins for cell cycle control; (e) therapeutic proteins; and (f) diagnostic proteins.

14: A polynucleotide according to claim 13, wherein said sequence from said another protein has the human/mammalian cell cycle-regulating function of p53 protein.

5 15: A polynucleotide according to claim 13 comprised in an open reading frame that is operably linked to a promoter sequence.

16: An expression vector comprising a polynucleotide according to claim 15.

10 17: A vector according to claim 16, comprising a polynucleotide according to claim 15 carried in a plasmid.

18: A vector according to claim 16, comprising a polynucleotide according to claim 15 carried in a virus vector or a non-viral transfection vector.

15

19: A method of inhibiting cell division, which comprises exposing a cell that comprises insufficient active/free p53 to arrest its cell cycle, to contact with a fusion polypeptide according to claim 5.

20 20: A method of inhibiting tumour cell division, which comprises exposing a tumour cell present in a tumour cell mass, said tumour cell comprising insufficient active/free p53 to arrest its cell cycle, to contact with a vector according to claim 9 encoding a fusion polypeptide according to claim 2, thereby causing said cell to express a fusion polypeptide according to claim 2 and to expose other cells of the tumour cell mass
25 to contact with a fusion polypeptide according to claim 2.

21: A mammalian or microbial host cell comprising a vector according to claim 16 or other polynucleotide according to claim 15.

22: A method of transporting a fusion polypeptide according to claim 1 to a target
5 population of cells, comprising: introducing a polynucleotide or other vector encoding the fusion polypeptide into a first part of the target population of cells, e.g. by transfection or microinjection; thereby expressing the encoding polynucleotide to produce the fusion polypeptide, thereby to cause the fusion polypeptide to be
10 exported from said first part of said target population, and to cause it to be taken up by a second part of the target population of cells not directly producing the fusion polypeptide.

23: A method of transporting a coupled polypeptide or fusion polypeptide according to claim 1 into a target population of cells, comprising directly exposing the
15 cells to a preparation of the polypeptide, to cause the target cells to take it up.

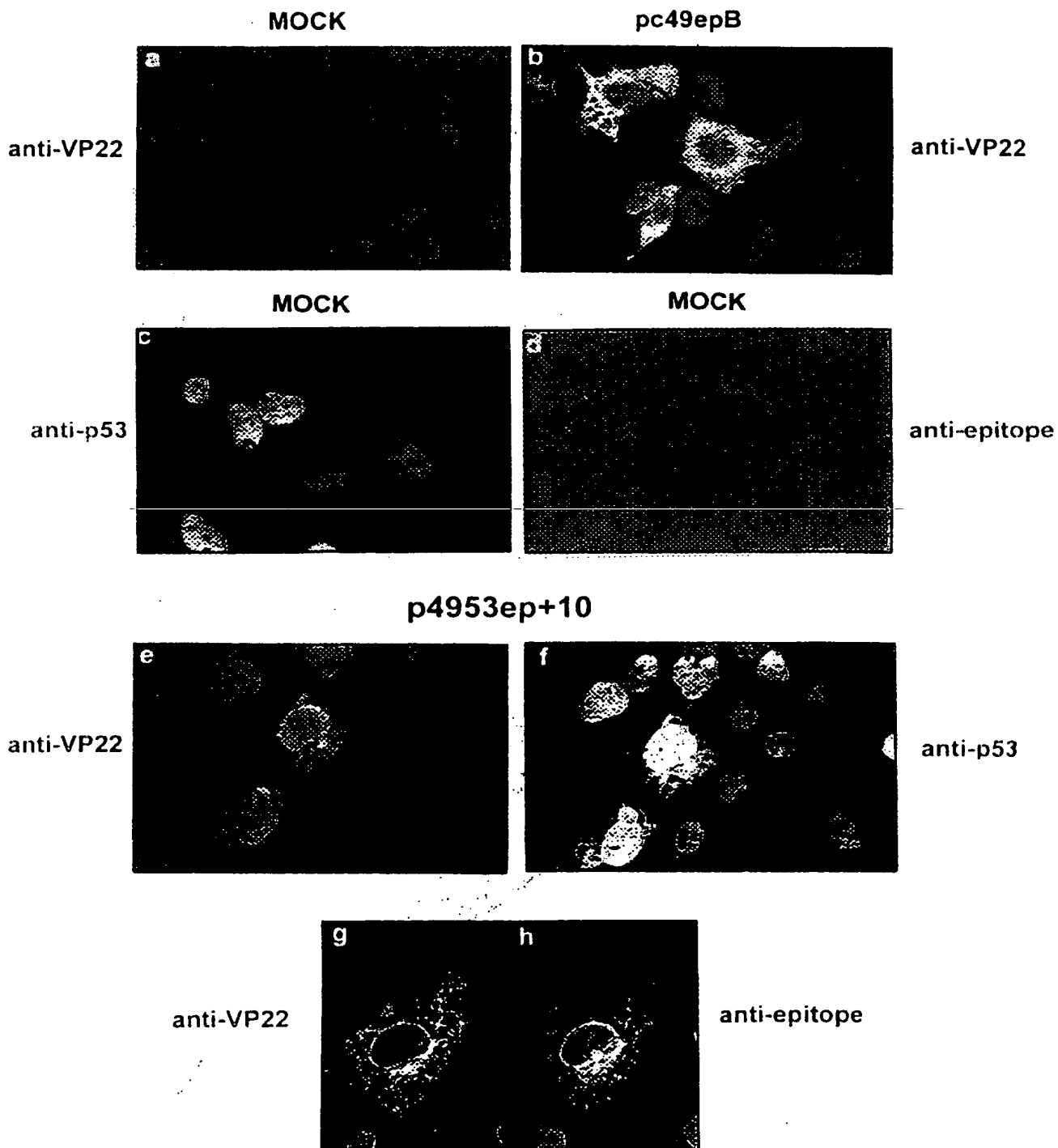
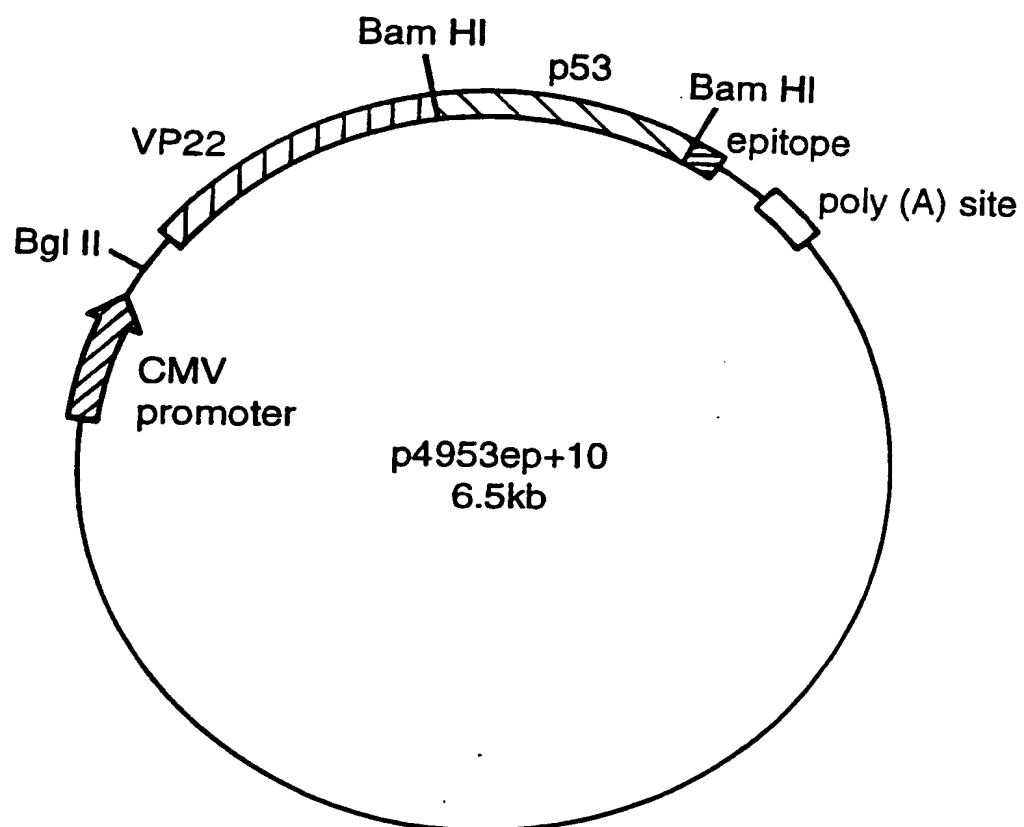
FIG. 1

FIG. 2

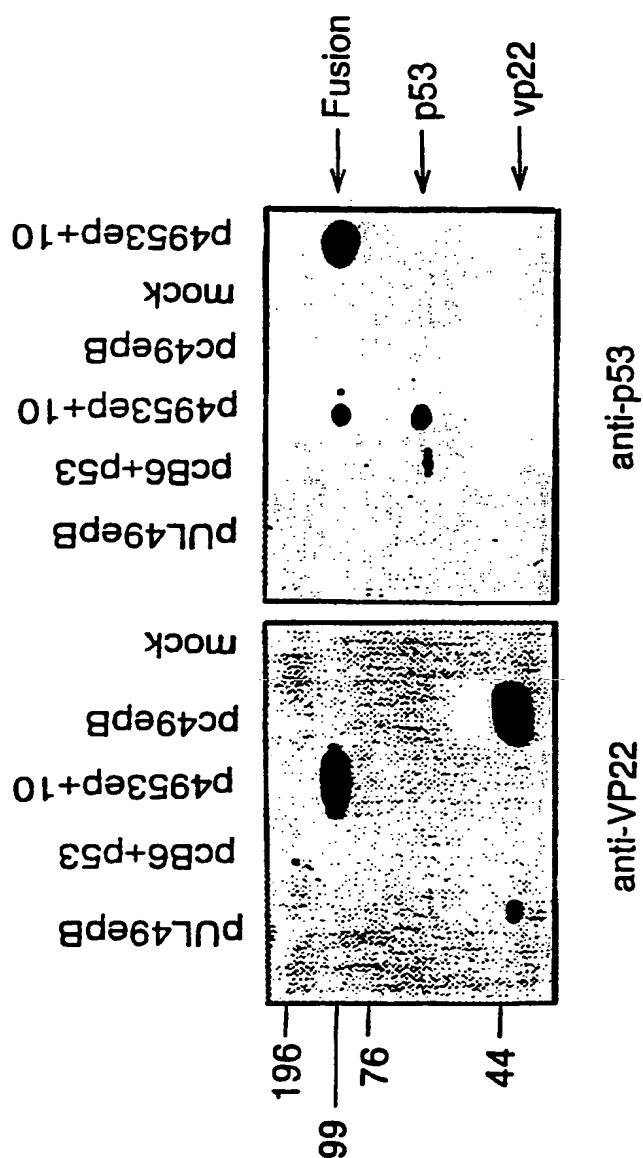


FIG. 3

INTERNATIONAL SEARCH REPORT

Int. Application No

PCT/GB 98/00207

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/62 C07K14/035 C07K14/47 C12N15/79

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 97 05265 A (O'HARE PETER FRANCIS JOSEPH O ;ELLIOTT GILLIAN DAPHNE (GB)) 13 February 1997 cited in the application see the whole document	1,13,22, 23
X,P	ELLIOTT G., O'HARE P.: "Intercellular trafficking and protein delivery by a herpesvirus structural protein" CELL, vol. 88, 24 January 1997, pages 223-233, XP002064725 cited in the application see the whole document	1,13, 15-21,23
-/-		

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

12 May 1998

Date of mailing of the international search report

27/05/1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Panzica, G

INTERNATIONAL SEARCH REPORT

Inter. Jnal Application No

PCT/GB 98/00207

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, P	WO 97 04092 A (RHONE POULENC RORER SA) 6 February 1997 see abstract see page 5, line 17 - page 12, line 20 ----	1-5, 13-23
A	EP 0 475 623 A (UNIVERSITY OF CALIFORNIA) 18 March 1992 see the whole document ----	1-6, 13-23
A	BAKER S J ET AL: "SUPPRESSION OF HUMAN COLORECTAL CARCINOMA CELL GROWTH BY WILD-TYPE P53" SCIENCE, vol. 249, no. 4971, 24 August 1990, pages 912-915, XP000293056 ----	1-6, 19, 20
A	MERCER W E ET AL: "WILD TYPE HUMAN P53 IS ANTIPROLIFERATIVE IN SV40-TRANSFORMED HAMSTER CELLS" ONCOGENE, vol. 5, no. 7, July 1990, pages 973-980, XP000619252 ----	1-6, 19, 20
A	SHAW P ET AL: "INDUCTION OF APOPTOSIS BY WILD-TYPE P53 IN A HUMAN COLON TUMOR-DERIVED CELL LINE" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 89, May 1992, pages 4495-4499, XP000619253 -----	1-6, 19, 20

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 98/00207

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9705265 A	13-02-1997	AU 6623996 A	26-02-1997
WO 9704092 A	06-02-1997	FR 2736915 A	24-01-1997
		AU 6618696 A	18-02-1997
		EP 0839194 A	06-05-1998
		NO 980203 A	10-03-1998
EP 0475623 A	18-03-1992	AT 136793 T	15-05-1996
		AU 8183694 A	23-02-1995
		AU 653356 B	29-09-1994
		AU 8262991 A	27-02-1992
		CA 2047090 A	25-02-1992
		DE 69118789 D	23-05-1996
		DE 69118789 T	26-09-1996
		EP 0710722 A	08-05-1996
		ES 2085966 T	16-06-1996
		FI 913978 A	25-02-1992
		JP 7059579 A	07-03-1995
		KR 9700189 B	06-01-1997
		US 5532220 A	02-07-1996

Form PCT/ISA/210 (patent family annex) (July 1992)